Online Resources

Visualization and Functional Analysis of the Oligomeric States of *Escherichia coli* Heat Shock Protein 70 (Hsp70/DnaK)

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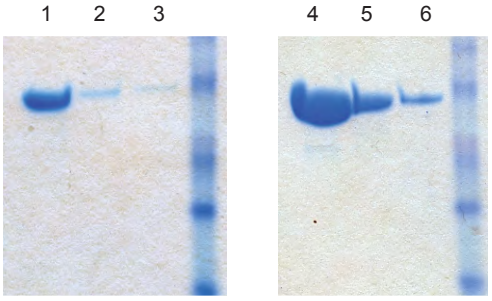
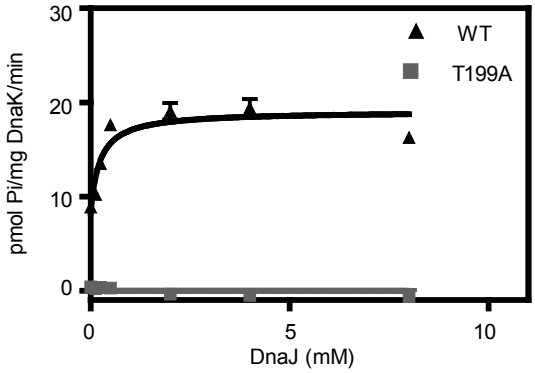
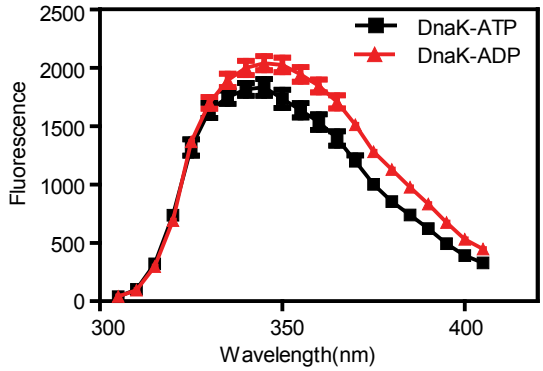
a) DnaK WT and T199A obtained at >90% purity

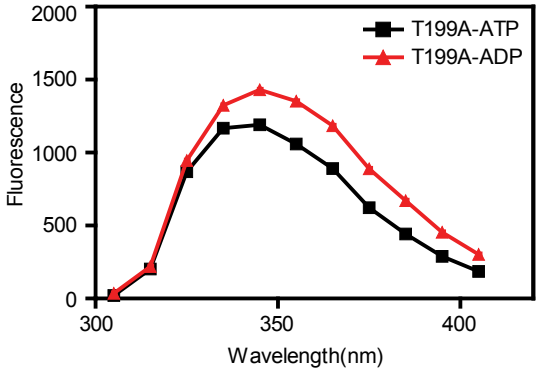


b) DnaK T199A is unable to hydrolyze ATP

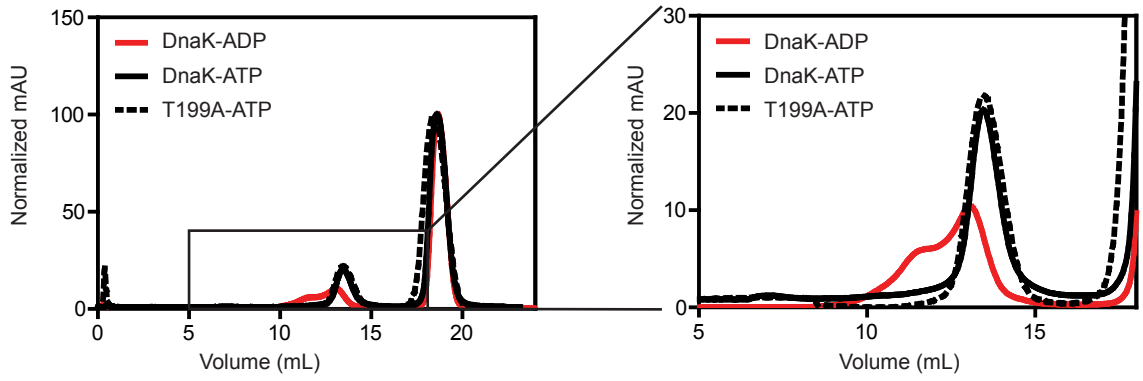


c) Characteristic change in trptophan fluorescence observed upon binding ATP

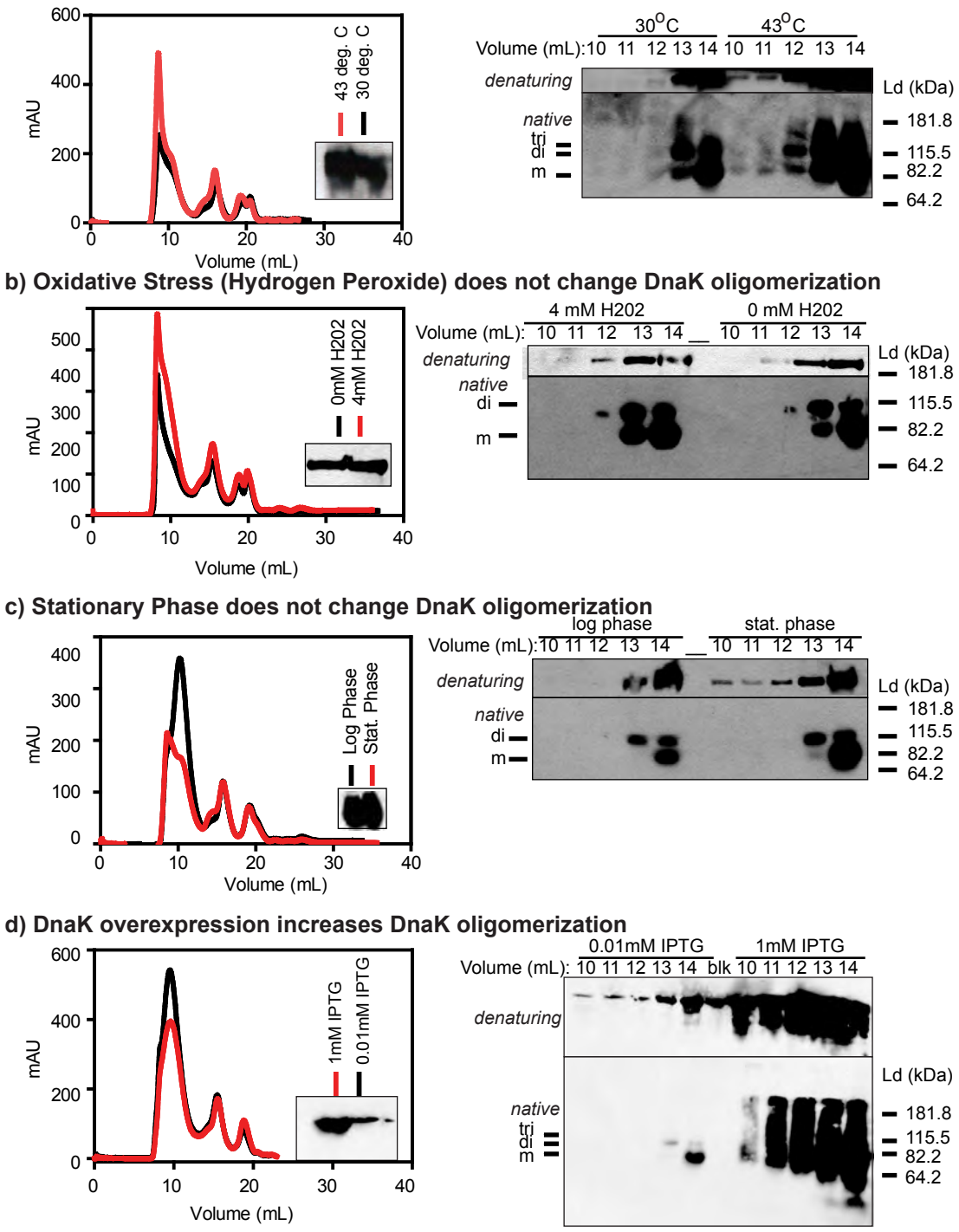




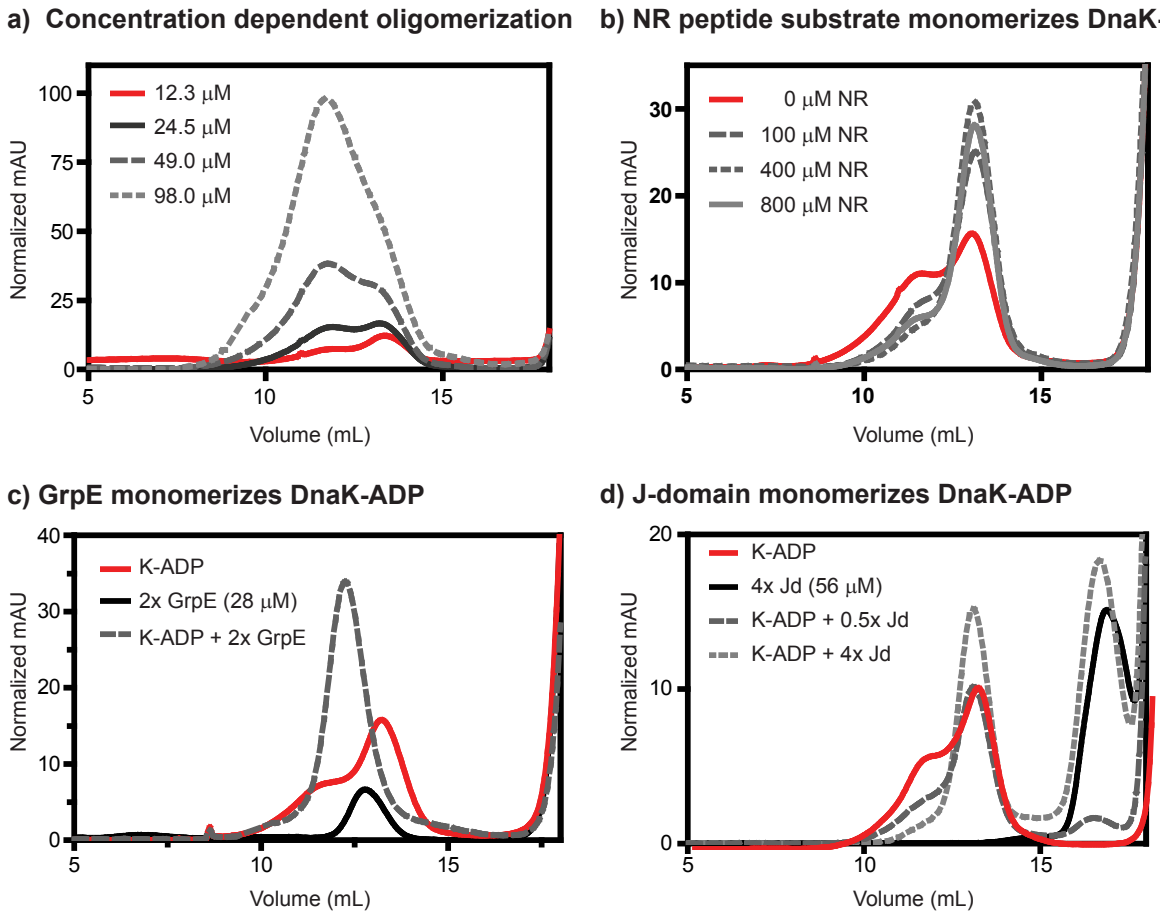
d) Zoom in on Full GF trace



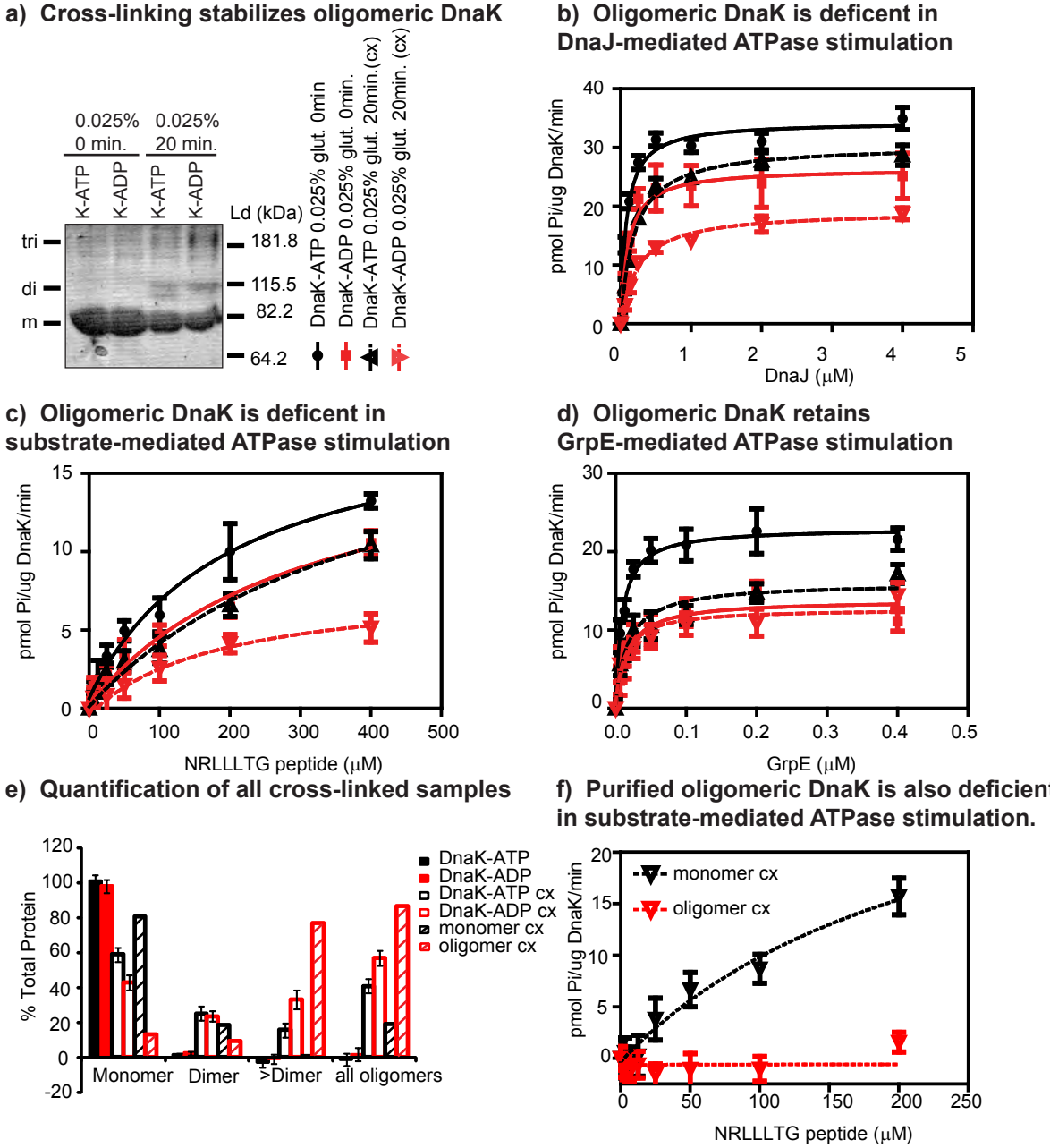
Online Resource 1 Protein Purification and nucleotide-state validation (A) A representative preparation of DnaK wild type is shown; lanes 1, 2,3 contain 1, 0.2, 0.1 μg of DnaK and demonstrate > 90% purity. Likewise, lanes 4,5,6 contain 7, 3.5, 0.7 μg of DnaK T199A and demonstrate >90% purity. (B) The DnaJ-mediated stimulation of DnaK's ATPase activity is shown for wild-type DnaK (WT) and a mutant DnaK (T199A), unable to hydrolyze ATP. (C) By tryptophan fluorescence, treatment of either DnaK WT or T199A with 1mM ATP or ADP for 30 minutes on ice resulted in the characteristic quenching of tryptophan fluoresence expected for the ATP-bound state. (D) A view of the full gel filtration trace from the experiment depicted in Figure 1 is provided. The box indicates the zoomed-in portion that is represented to the right and in Figure 1A. Just to the right of the zoomed-in portion is the major peak created by elution of nucleotide. This peak was used to normalize the mAU and control for any variations in the amount of sample injected onto the column.



Online Resource 2 DnaK-oligomerization within *E. coli* under various stress-related conditions
(a) Gel filtration curves of cellular lysates are shown to represent the total protein present in the cellular lysate tested. The gel insert depicts western blot analysis of the amount of DnaK present within total cell lysate by denaturing gel electrophoresis. Both denaturing and native gel electrophoresis was performed on fractions collected from gel filtration (as in Figure 1). Dnak was probed by western blot. Heat shock resulted in an increase in DnaK present in earlier fractions. Further bands consistent with the molecular weight of trimeric (tri), dimeric (di), and monomeric (m) DnaK were observed by native gel. The sample depicts a duplicate experiment. (b) Likewise the effect of oxidative stress, using hydrogen peroxide, stationary phase (c), and over-expression of DnaK (d) on self-oligomerization were evaluted. Experiments were performed in duplicateand a representative result is shown for each condtion. No increase in DnaK self-oligomerization was observed under oxidative stress or during stationary phase. However, a dramatic increase in DnaK present in early fractions was observed when DnaK was over-expressed. This is suggestive of increased oliogmerization.

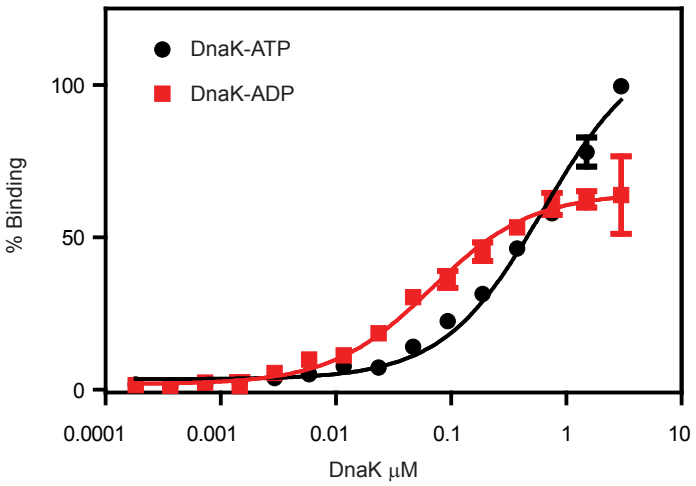


Online Resource 3 Co-chaperones and substrate monomerize DnaK-ADP
(a) DnaK oligomerization is concentration dependent. Gel filtration was performed using increasing concentrations of purified DnaK with 1 mM ADP. Addition of either NRLLLTG peptide substrate (b), GrpE (c), or the J-domain of DnaJ (Jd) (d) lead to monomerization of 14 μM DnaK-ADP (K-ADP). In the case of GrpE, DnaK forms a complex with the dimeric GrpE, giving a single symmetrical peak. It is interesting to note that full length Ydj1, a yeast DnaJ homolog, has been shown to induce DnaK oligomerization in the presence of ATP but not ADP[35]. However, this oligomerization resulted in higher order species than observed in the ADP bound state of DnaK. Thus, King et al. proposed that Ydj1 promoted DnaK self-assembly by utilizing the substrate binding capabilities of Ydj1 to present one DnaK to another, as DnaJ typically presents another client protein to DnaK. Our data shows that the J-domain of DnaJ promotes the monomerization of K-ADP (d). This results supports the proposed model of King et al. and suggests that the self-oligomerization promoted by the ADP bound state is distinct from the self-oligomerization promoted by DnaJ in the presence of ATP.

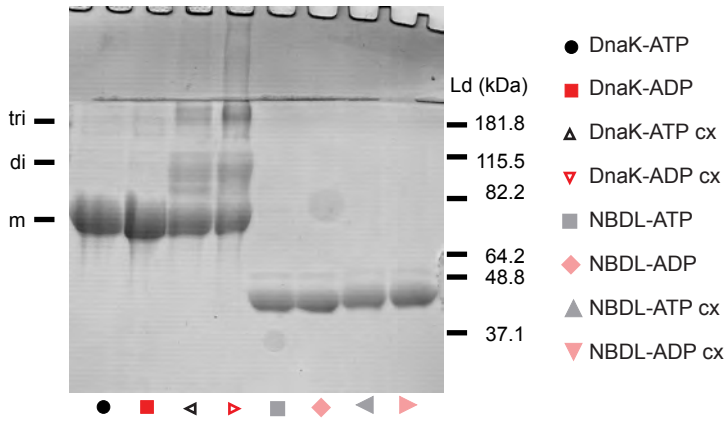


Online Resource 4 Examing the ability of co-chaperones to stimulate the ATPase rate of Oligomeric DnaK
(a) Stabilization of DnaK oligomers by chemical cross-linking. DnaK (K) was pre-incubated with either ATP or ADP and treated with 0.025% glutaraldehyde (glut.). Samples were either incubated for 20 min to allow cross-linking (cx) or quenched (0 min) immediately. Bands consistent with monomeric (m), dimeric (di) and trimeric (tri) DnaK were observed, as well as higher-order structures, The ATPase rate ofthese samples were then tested in the presence of DnaJ (b), NRLLTG peptide substrate (c), and GrpE inthe presence of DnaJ 0.5μM and NRLLLTG substrate 100μM (d). All of the experiments were performed in triplicate and the error bars represent the standard error of the mean. A negative control lacking DnaK was also tested and subtracted in each experiment. Finally, in this figure, all curves are baseline normalized so all samples started at 0. Graphs thus reflect only increase in ATPase rate mediated by co-chaperone not overall ATPase rate. We observe that cross-linked DnaK-ADP has a decreased ability to be stimualted by DnaJ, consistent with the results observed in Figure 2C. Values reported in table one were obtained from fitting all available data from Figure 2 and the above experiment. Further we observe that oligomeric DnaK (K-ADP cx) also has a decreased ability to be stimulated by peptide substrate. However, GrpE-mediated stimulation seems to be unaffected under the given cross-linking conditions. (e) The gels shown in Figure 2A and (a) are representative of nine cross-linking reactions performed. Quantification ofbands was done by image J on all cross-linked samples and reveal that the cross-linked DnaK-ADP samplehas more oligomeric species than the cross-linked DnaK-ATP sample (57+/-5 % compared to 40+/-4%). Toensure that the differences in activity observed between the cross-linked DnaK-ATP sample and DnaK-ADP sample were in fact due to the increase in oligomeric species we purified oligomeric DnaK from monomeric DnaK from a cross-linked DnaK-ADP sample (see Figure 2E for the gel). Quantification revealed that we obtained a sample that was >80% monomeric (monomer cx) and a sample that was >80% oligomer (oligomer cx). We demonstratedthat the oligomeric sample was deficient in DnaJ-mediated ATPase stimulation (Figure 2F) and substrate-mediated ATPase stimulation (f).

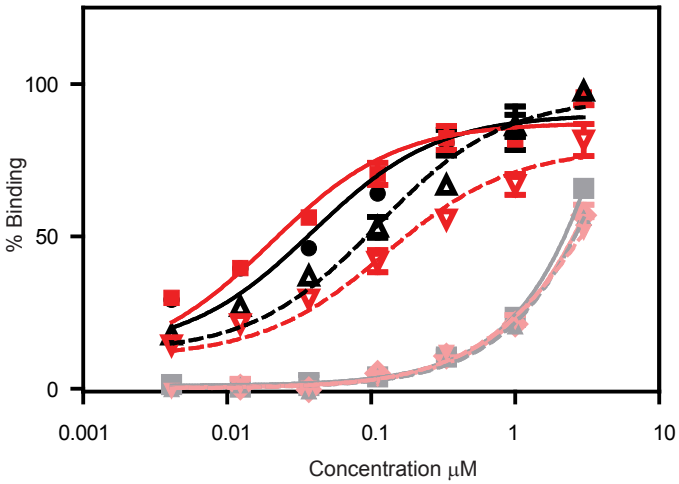
a) Nucleotide dependence of substrate binding



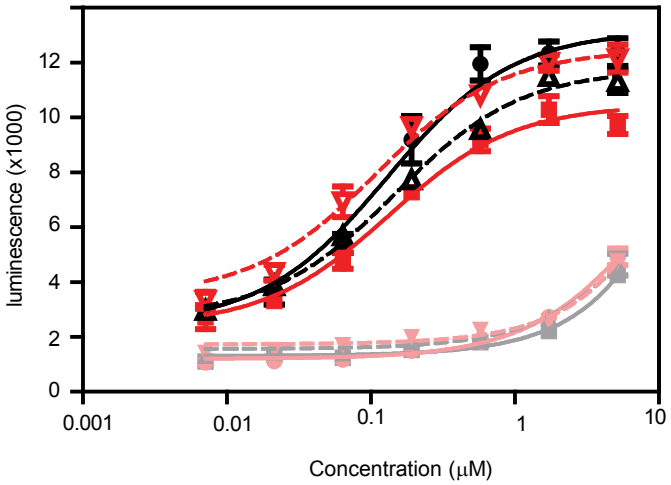
b) Cross-linking reaction of DnaK and NBDL of DnaK



c) Non-specific Binding of NBDL is low within the luciferase binding assay

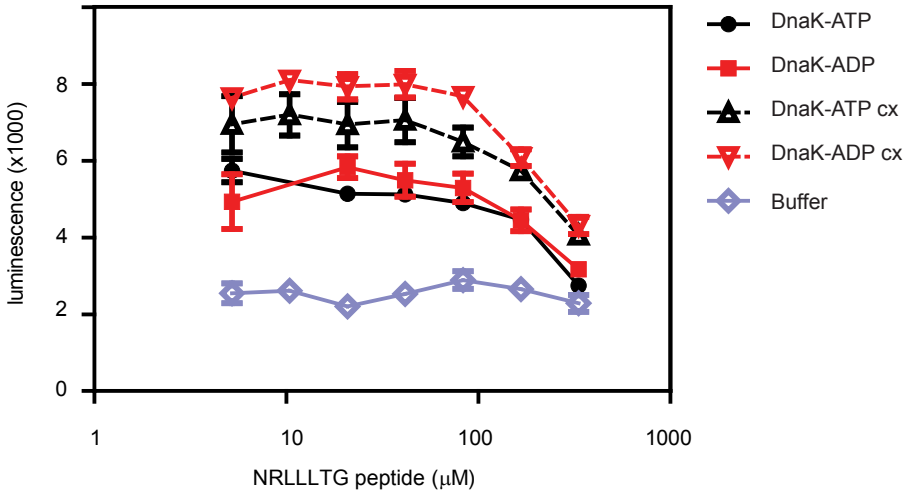


d) Non-specific Binding of NBDL is low within the holdase assay



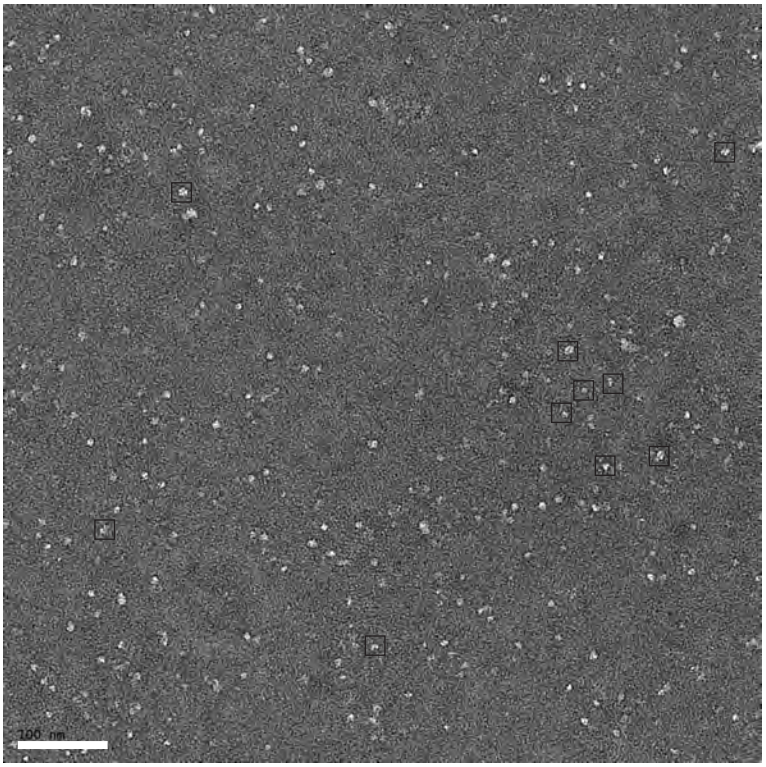
Online Resource 5 Luciferase binding and holdase activity is mediated through the substrate binding domain in both cross-linked and non-crosslinked samples of DnaK

(a) In the ELISA based susbtrate binding platform, DnaK-ATP binds with a Kd,app of 0.7 +/- 0.1 μM and DnaK-ADP binds with a Kd,app of 0.07 +/- 0.01 μM. When testing cross-linked samples, all samples were diluted in the presence of 1mM ATP to minimize effects of pre-incuptation with ADP or ATP. (b) We cross-linked the nucleotide binding domain with linker of DnaK (NBDL) under the same cross-linking conditions used for DnaK. The gel shows that under these condtions NBDL is primarily monomeric and cross-linking does not stabilize oligomeric forms of NBDL. (c) We tested the ability of these samples to bind luciferase, we found that NBDL interacts with luciferase non-specifically with a Kd > 3 μM. Our results also show that the cross-linking reaction does not increase this non-specific binding interaction appreciably. (d) Similar results were obtained in the holdase assay.

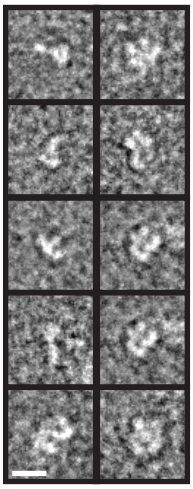


Online Resource 6 NRLLLTG peptide can inhibit DnaK mediated holdase activity
The NRLLLTG peptide is known to interact in the substrate binding pocket of DnaK and we observe that it can inhibit the holdase activity of DnaK to an equal extent in both crosslinked and non-crosslinked samples. This result further supports the idea that the crosslinked samples bind substrate via the substrate binding domain.

a) Raw Image DnaK-ADP

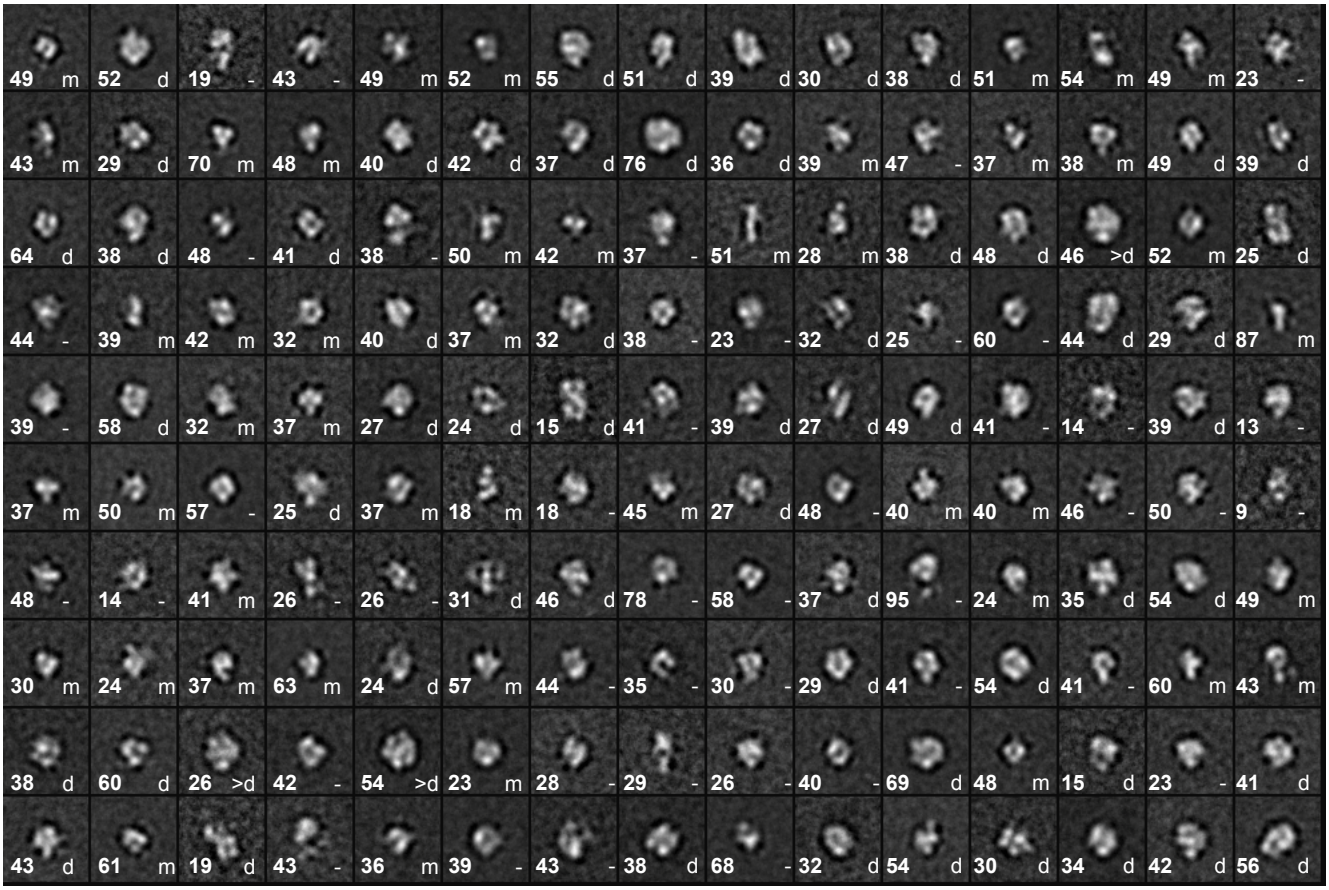


b) Raw Particle Images



10 nm

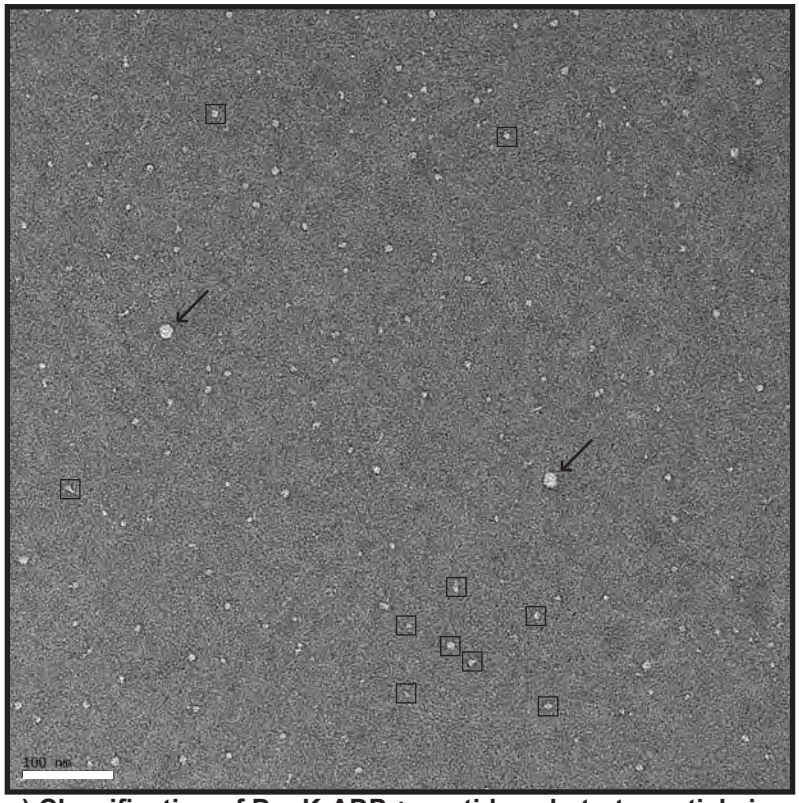
c) Classification of DnaK-ADP particle images



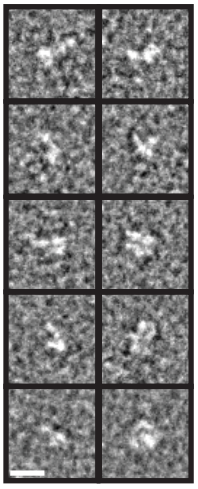
10 nm

Online Resource 7 2D projection analysis of DnaK-ADP (a) A raw image of DnaK-ADP embedded in negative stain is shown. Boxes are meant to higlight some of the particles selected for 2D averaging. (b) Examples of raw particle images used for 2D classification are shown. (c) Classification of DnaK-ADP particle images. 6,090 particle images were classified into 150 classes. The numbers in each box indicate the number of particles making up each class average and (m), (d), (>d), (-) indicate the designation assigned by the authors as monomer, dimer, greater than dimer, and unassigned, respectively.

a) Raw Image DnaK-ADP + peptide substrate

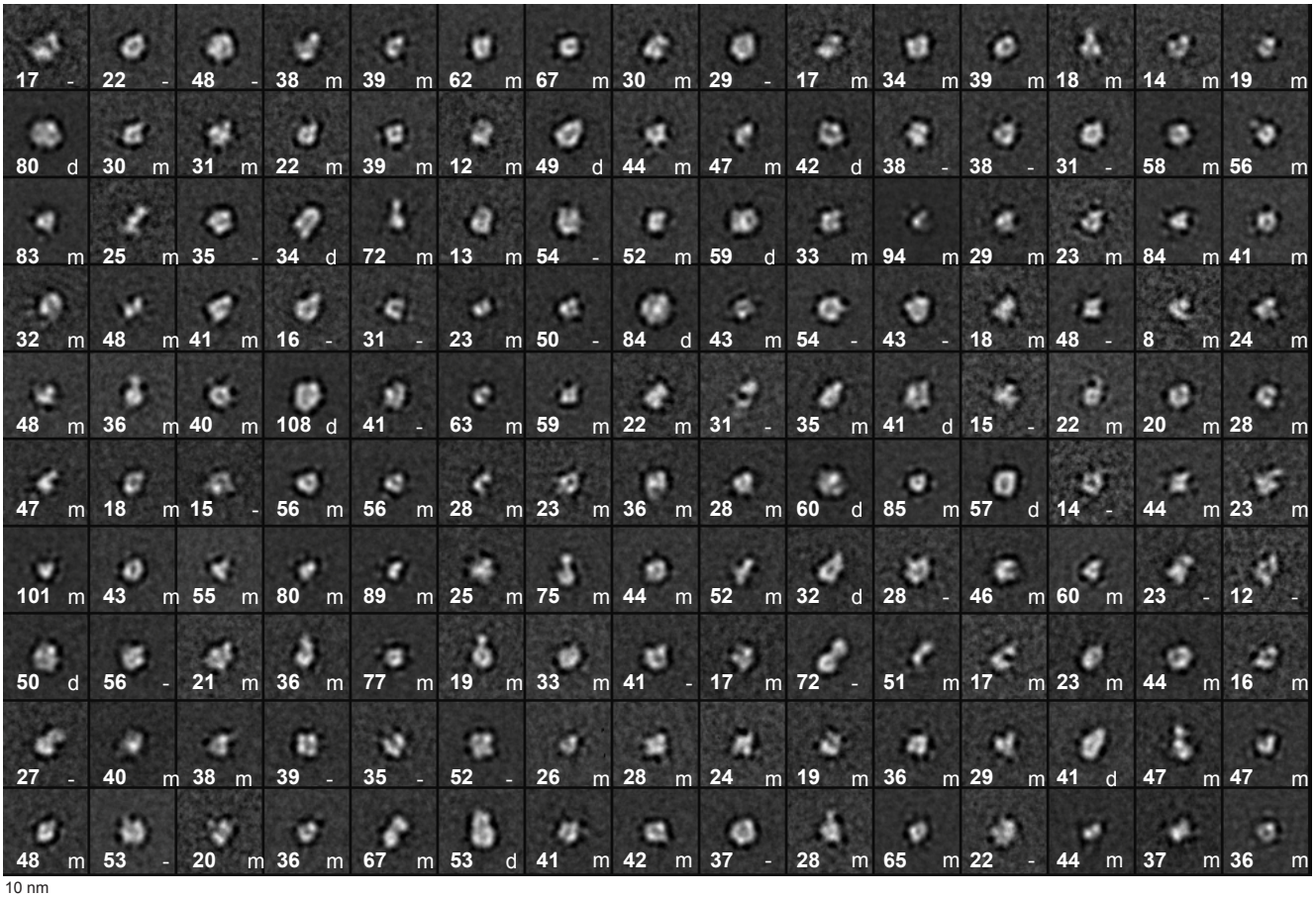


b) Raw Particle Images



10 nm

c) Classification of DnaK-ADP + peptide substrate particle images



10 nm

Online Resource 8 2D projection analysis of DnaK-ADP with peptide (NRLLLTG) substrate (a) A raw image of DnaK-ADP + peptide substrate embedded in negative stain is shown. Boxes are meant to higlight some of the particles selected for 2D averaging. (b) Examples of raw particle images used for 2D classification are shown. (c) Classification of DnaK-ADP with peptide substrate particle images. 6,090 particle images were classified into 150 classes. The numbers in each box indicate the number of particles making up each class average and (m), (d), (>d), (-) indicate the designation assigned by the authors as monomer, dimer, greater than dimer, and unassigned, respectively.